American Journal of Hematology 51:276–281 (1996)

Hemoglobin Debrousse (β96[FG3]Leu → Pro): A New Unstable Hemoglobin With Twofold Increased Oxygen Affinity

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Hemoglobin Debrousse (β96[FG3]Leu → Pro) is a new unstable variant, with high oxygen affinity responsible, in the steady state, for an apparently well-compensated chronic hemolytic anemia. The functional properties of this variant are due to the replacement of a leucine residue which is involved in the hydrophobic environment of the proximal side of the heme. This electrophoretically neutral hemoglobin was found as a de novo case in a 6-year-old girl suffering from severe anemia with hemolysis and transient aplastic crisis, following infection by parvovirus B19. © 1996 Wiley-Liss, Inc.

Key words: unstable hemoglobin, parvovirus B19, hemolytic anemia, transient aplastic anemia, high oxygen affinity hemoglobin

INTRODUCTION

In the case of moderately unstable hemoglobins (Hb), several circumstances may render the diagnosis difficult [1]. For instance, the anemia may be apparently well compensated, the Hb variant may be electrophoretically neutral, and a familial history of hereditary hemolytic anemia may be absent. In these variants, the structural modification may affect regions crucial both for the stability of the molecule and for oxygen binding. As a consequence, oxygen delivery to the tissues is sufficiently impaired to stimulate erythropoiesis further and, thus, to increase Hb levels to values very close to the normal. The presence of such an unstable Hb may be suggested by a persistent chronic mild hemolysis in the steady state but becomes much more suggestive when administration of an oxidative drug or infectious episodes lead to hemolytic crises. In many cases the presence of the unstable variant remains unknown until such a complication has occurred.

We report here on Hb Debrousse (β96[FG3]Leu → Pro), a new moderately unstable hemoglobin variant that was found, as a de novo case, during a severe anemic episode due to an infection by parvovirus B19. In these circumstances the hemolytic process was associated with a transient aplastic anemia.

MATERIALS AND METHODS

Hematological and Electrophoretic Studies

Blood samples were collected in EDTA, and hemolysates were prepared by lysis of the washed erythrocytes with 4 vol of water and 0.5 vol of toluene, followed by centrifugation. Hematologic studies were performed by routine methods.

Methods for electrophoretic analysis of hemoglobin included electrophoresis on cellulose acetate at alkaline pH, citrate agar electrophoresis, isoelectric focusing (IEF) of hemoglobin, electrophoreses of globin chains in urea at pH 6.0 and 9.0, or in the presence of Triton X-100 [2,3]. The percentages of the various hemoglobin fractions (except for Hb Debrousse) were measured by ion-exchange high-performance liquid chromatography (HPLC) (BioRad, Hercules, CA). Stability was assayed by the isopropanol test [4]. The activities of the red blood cell (RBC) enzymes were estimated by routine techniques.

Received for publication June 30, 1995; accepted November 8, 1995.

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TABLE I. Hematological Data

	Steady state (Feb. 1993)	Parvovirus B19 infection (1992)	Hemolytic crisis due to infectious episodes (Dec. 1993)	(1995)
RBC (10^{12}/L)	4.47	1.78	3.72	3.08
Hb (g/dl)	13.0	5.0	11.3	8.8
MCV (fl)	89	95	99	91
Reticulocytes (10^9/L)	174.0	35.6	230	197
Serum bilirubin (μmol/L)		37		54

TABLE II. Electrophoretical Parameters

Hb	IEF[a]	Cellulose-acetate[b] (pH 8.6)	Citrate-agar	Urea[c] (pH 9.0)	pH 6.0	Triton X-100
Hb A	0.0	0.0	0.0	20	20	20
Hb Debrousse β96 (FG3) Leu → Pro	−0.8	0.0	0.0	20	20	19

[a]In mm from Hb A in the IEF reference map [3].
[b]According to a comparative scale [2].
[c]Values are given only for the β-chain.

Structural Characterization

Determination of the structural abnormality was carried out as previously described [5]. Although detectable by IEF, the abnormal hemoglobin fraction was electrophoretically neutral and could not be separated from Hb A. Globin was therefore prepared from the whole lysate. The polypeptide subunits were separated by reverse-phase HPLC (RP-HPLC), using a Brownlee C4 column (Brownlee Laboratories, Santa Clara, CA) eluted at a flow rate of 1 ml/min by a gradient of acetonitrile from 38% to 41% in a solution containing 0.1% trifluoroacetic acid (TFA). After aminoethylation, globin chains were digested by trypsin, and the resulting peptides were separated by RP-HPLC using a Vydac C8 column (25 × 0.46 cm) (The Separation Group, Hesperia, CA). The amino acid compositions of the peptides were determined by RP-HPLC after hydrochloric acid hydrolysis and precolumn derivation into phenylthiocarbamyl amino acids.

Functional Studies

Oxygen binding properties were studied on the total lysate stripped of anions by passing it over a mixed ion-exchanger column. Oxygen equilibrium curves were performed at 25°C with an automatic, continuous method (Hemox-Analyzer) as previously described [6]. The buffer was 50 mM bis-Tris to which 50 μM Na EDTA and catalase (20 μg/ml) were added, to limit methemoglobin formation. Hemoglobin concentration was 60–80 μM on a heme basis. The oxygen equilibrium data were analyzed as previously described [6]. Analyses of the oxygen binding data were made within the framework of the two-state allosteric model (Monod-Wyman-Changeux model). The oxygen equilibrium curve of the proposita's hemolysate was fitted to the equation for Y, assuming a sum of two independent curves (65% Hb A and 35% Hb Debrousse, in the absence of significant amount of $\alpha_2\beta^X\beta^A$ hybrid tetramers), using an iterative nonlinear least-squares program. This allowed for calculation of the oxygen affinity parameters for the pure mutant hemoglobin.

DNA Studies

DNA was isolated from the leukocytes and spermatozoa using InstaGene Purification matrix (BioRad). A 866-bp fragment from the β-gene, including exons 1 and 2, was polymerase chain reaction (PCR)-amplified using the experimental conditions described below. The primers used were 5′TGTACTGATGGTATGGGGC3′ located 225 nucleotides (nt) upstream to the cap site and 5′AAACGATCCTGAGACTTCC3′ located in the second intron, 641 nt downstream to the cap site. The reaction mixture (30 μl) contained 100 ng of each primer, 200 μM of each dNTP in Taq polymerase buffer (20 mM Tris–HCl, pH = 8.40, 50 mM KCl, 1.5 mM $MgCl_2$) with 2 units of Taq polymerase (Gibco-BRL, Gaithersburg, MD). Thirty-three cycles of amplification were performed with denaturation at 95°C (1 min), annealing at 64°C (1 min), and extension at 72°C (2 min). The 866-bp amplified fragment was digested with *AluI* (Eurogentec, Serain, Belgium) for 2 hr at 37°C and with *AciI* (New England Nuclear, Beverly, MA) for 2 hr at 37°C. Digestion fragments were separated by agarose electrophoresis (3–4%

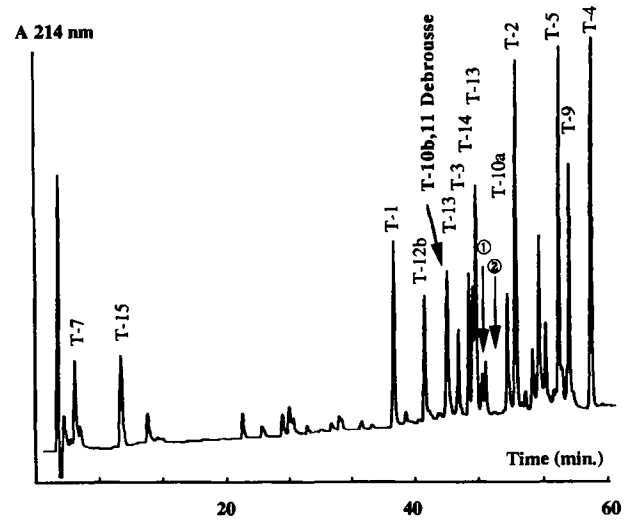

Fig. 1. Elution pattern of the tryptic digest of the aminoethylated β-chain of Hb Debrousse. Experimental conditions: 0.2 mg tryptic digest was applied on a Vydac C8 column (25 × 0.46 cm), elution was obtained at a flow rate of 1.5 ml/min using a curvilinear gradient between solvent A (TFA 0.2% in water) and solvent B (acetonitrile 70%/TFA 0.2% in water) from 0% to 60% B in 60 min. Positions of missing peptides βT-11 and βT-10 are indicated by arrows ① and ②, respectively. Peptide βT-10a is present and an abnormal peak is eluted between βT-12b and βT-13.

agarose), stained with ethidium bromide, and visualized under UV light as previously described [7].

RESULTS

Case Report and Hemoglobin Study

The proband, a 6-year-old girl whose father was from Sicily and whose mother was from Corsica, was hospitalized in 1992 for a febrile pharyngitis and treated with cephalosporin. This episode was followed by an acute anemia. The girl presented with a cutaneous pallor and a scleral icterus. Physical examination showed splenomegaly and hepatomegaly (5 and 2 cm below costal margin, respectively). Hematological data showed a hemolytic anemia with a low reticulocyte count (Table I). Serum bilirubin was elevated (total: 37 μmol/L; conjugated: 14 μmol/L). Hemoglobinuria without hematuria was noted. The serum ferritin level was 190 ng/ml. An erythroblastosis indicative of a spontaneous recovery was noticed on the bone marrow smears. Eight days after the febrile episode, the reticulocyte count was at 479 × 10^9/L. The patient received no blood transfusion. Three weeks later, the presence of IgM specific for parvovirus B19 infection was detected. A systematic Hb study by IEF revealed the presence of a Hb variant. In February 1993, when the proposita was seen again, the physical examination showed scleral subicterus, cutaneous pallor, and mild splenomegaly. Hematological data were of a well-compensated chronic hemolytic anemia (Table I). The proband was seen later, in December 1993 and in 1995, when she suffered from hemolytic crisis induced by infectious episodes (Table I).

The pattern of electrophoretic mobilities of the Hb variant in the various experimental conditions used for presumptive diagnosis is shown in Table II. These mobilities are very close to those of Hb A, except for IEF and urea–triton polyacrylamide gel electrophoresis (PAGE). IEF also revealed the presence in trace amounts of several slow migrating bands that may correspond to partly oxidized or deheminized derivatives. Hb A_2 and Hb F were within the normal range (Hb A_2 = 3.5%, Hb F = 0.9%).

Isopropanol stability test was positive: 17% of the hemolysate was precipitated after 10 min versus 2% in a

TABLE III. Amino Acid Composition and Sequence of the Uncleaved βT10b-11 Peptide of Hb Debrousse

		Expected for	
Residues	Found	βT-10b	βT-11
Aspartic acid	3.0	1	2
Glutamic acid	1.2		1
Histidine	0.9		1
Arginine	0.8		1
Proline	**2.1**		**1**
Valine	0.9		1
Leucine	**0**		**1**
Phenylalanine	0.8		1
Lysine	0.9	1	

	T-10a T-10b T-11
	93▼ 94 ▼ 96
HbA	-Cys-Asp-Lys-**Leu**-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-
	93▼ 94 96
Hb Debrousse	-Cys-Asp-Lys-**Pro**-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-

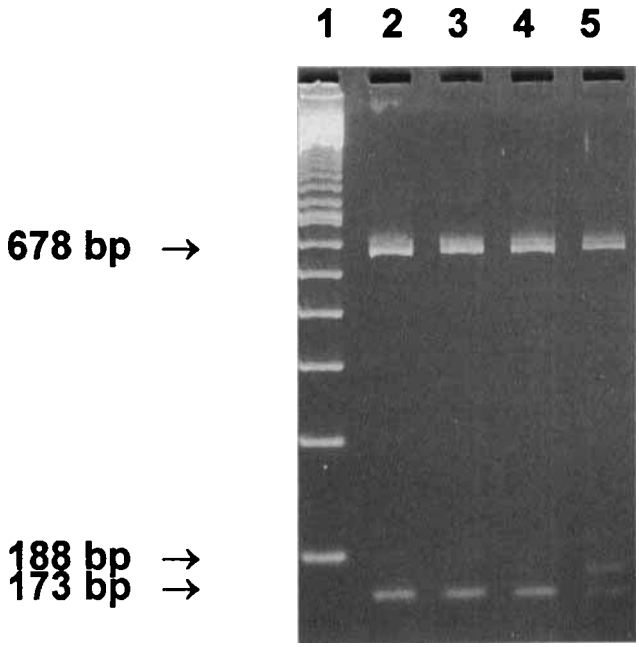

Fig. 2. Agarose electrophoresis of the *AluI* fragments from 866 bp PCR-amplified fragment. Lane 1: DNA ladder; lane 2, proposita's father (leukocytes); lane 3, proposita's father (spermatozoa); lane 4, proposita's mother; lane 5, proposita.

control. Studied RBC enzymes were normal. The parents of the proposita displayed no abnormal clinical or hematological features. Their hemoglobin IEF was also normal.

Structural Characterization

An abnormal β-chain, amounting to approximately 35% of the total of the β-chains, was eluted before the normal one during RP-HPLC on a Brownlee C4 column. After aminoethylation, which converts the cysteine into aminoethylcysteine residues that provide additional cleavage points for trypsine, the elution pattern of the tryptic digest showed several differences with the normal. Peptide βT-10, which ends at lysine 95, and peptide βT-11 were missing, but peptide βT-10a, which ends at aminoethylcysteine 93, was still present. In addition, an abnormal peak was found that eluted between βT-12b and βT-13 (Fig. 1). The amino acid composition of this peak showed that it was an uncleaved peptide containing βT-10b and βT-11 (sequence 94–104) (Table III). In this peptide, the single leucine residue, located at the N-terminus of βT-11, was missing and was replaced by a proline (Table III). The presence of a proline at the C-terminal of a lysine is known to impair the tryptic cleavage at this point and to explain the changes observed in the chromatogram. We propose the name of Hb Debrousse, from the place where it was found, for this new variant having the substitution β96 (FG3) Leu → Pro.

DNA Studies

Digestion of the 866-bp PCR-amplified normal fragment by *AluI* yields three fragments: 678, 173, and 15 bp. As shown in Figure 2, the substitution CTG → CCG suppressed the second restriction site for *AluI*, giving a restriction fragment of 188 bp. In addition, the substitution CTG → CCG created an *AciI* restriction site that yielded two fragments: 694 and 172 bp (not shown).

The substitution CTG → CCG was detected neither in the DNA extracted from leukocytes of the proposita's parents nor from that of the father's spermatozoa. Nonpa-

TABLE IV. Oxygen Binding Parameters for Hb Debrousse*

Experimental conditions	HbA		Hemolysate Hb A/Hb Debrousse		Hb Debrousse (calculated)[a]	
	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}
pH 7.2	4.5	2.8	3.7	2.0	2.2	2.1
pH 7.2 + 2,3-DPG 1 mM	14.0	2.9	11.2	2.2	7.0	2.6
pH 6.5	10.5	2.6	8.6	2.2	5.2	2.4

	Calculated heterotropic effects	
	Hb A	Hb Debrousse
DPG effect: ($\Delta\log P_{50} \pm 1$ mM 2,3-DPG)	0.49	0.50
Bohr effect: ($\Delta\log P_{50}/\Delta pH$)**	−0.54	−0.53

*Other conditions: NaCl 0.1 M, Bis Tris 0.05 M, catalase 20 μg/ml, EDTA 50 μM, 25°C.

[a]The oxygen dissociation curves of Hb Debrousse was calculated by the nonlinear least-squares procedure, assuming the presence of 35% Hb Debrousse in the lysate and using an algebraic sum of two MWC allosteric model curves.

[b]Bohr effect was estimated from measurements done at pH 6.5 and 7.2.

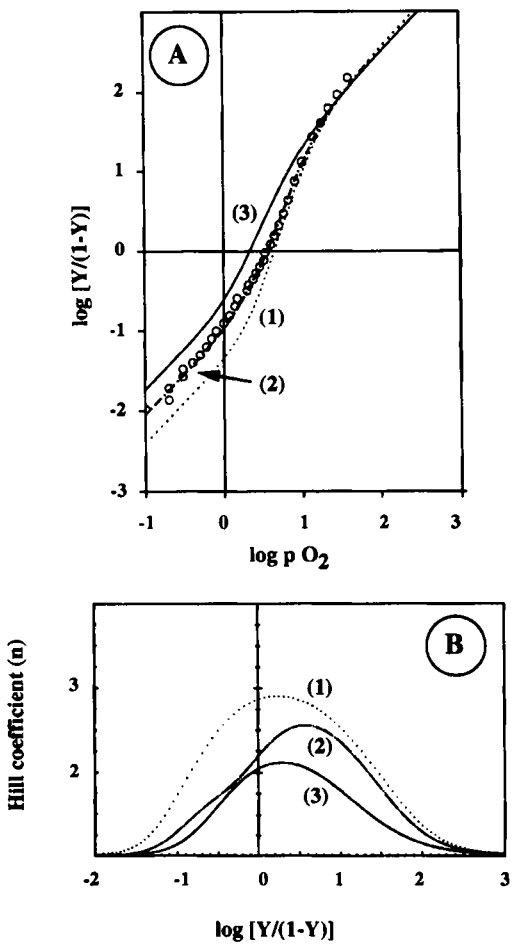

Fig. 3. Oxygen equilibrium curves. Experimental conditions: bis Tris 0.05 M, NaCl 0.1 M, pH 7.2, 25°C. A: Hill plot: (1) control curve for Hb A; (2) hemolysate Hb A/Hb Debrousse: symbols represent 1 over 6 experimental points, dotted line is the calculated curve resulting from the sum of the two individual curves using as allosteric parameters L = 8 × 10^4, c = 0.010, K_R = 0.25 mmHg and K_T = 25 mm Hg for Hb A and L = 7 × 10^3, c = 0.040, K_R = 0.22 mm Hg and K_T = 5.5 mmHg for Hb Debrousse; and (3) calculated curve for Hb Debrousse. B: Oxygen cooperativity curve. Curves represent the first derivative of the Hill equation.

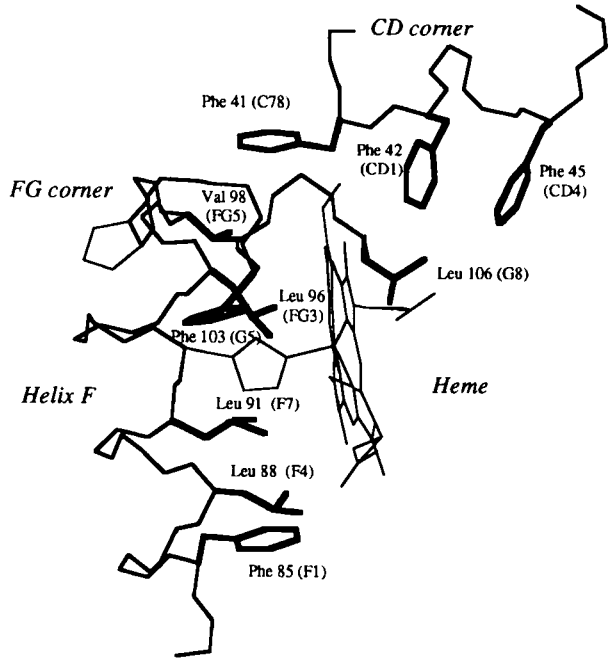

Fig. 4. Hydrophobic residues in interaction with the heme. In normal deoxyHb, several hydrophobic residues are in contact with the heme group. Bold lines, those located in the CD and FG corners and in helix F. These residues maintain the heme in the positions required for oxy or deoxy structure. Drawn using coordinates from file 3HHB (Fermi and Perutz, 1984) from the Protein Data Bank, Brookhaven National Laboratory.

ternity was ruled out using the forensic DNA amplification (HLA typing, class II, CRTS, Lyon). The following alleles were found: DRB 1: 1–7 and DQB 1: 0501–0201 for the proposita; DRB 1: 11–7 and DQB 1: 0301–0201 for the father, and DRB 1: 1 and DQB 1: 0501 for the mother (not shown).

Functional Studies

The oxygen equilibrium curves of the lysate showed, in addition to Hb A, the presence of a high oxygen affinity component (Table IV; Fig. 3A). Under all experimental conditions, the p50 of the hemolysate showed a 30–40% increase in oxygen affinity. The oxygen cooperativity curve (Fig. 3B), the first derivative of the Hill plot, was biphasic, but the abnormal component retained cooperativity with n values between 2.1 and 2.6. These data were consistent together with a twofold increased oxygen affinity of the pure abnormal component, relative to Hb A, and normal 2,3-DPG and Bohr effects.

DISCUSSION

The structural modification of Hb Debrousse affects a region that is important for the stability of the molecule and for oxygen binding [8]. The leucine residue at position FG3 is a heme contact directly involved in the hydrophobic environment of the proximal side of the heme, which includes phenylalanines 85 and 103 (Fig. 4). Hb Regina, another variant of position β96 (FG3), displays also a twofold increase in oxygen affinity [9]. In this case, the leucine residue is replaced by a valine: this residue, having a smaller side chain, is also likely to prevent the occurrence of the hydrophobic contact that contributes to maintain the heme in its right position. The stability of Hb Regina being normal, the clinical picture was that of a mild erythrocytosis with 25% increase of the blood volume instead of a hemolytic anemia [9]. Other stable Hb variants with high oxygen affinity have been described affecting the proximal side of the heme. Among those, a

good example is provided by Hb Saint Nazaire (β103 Phe → Ile), whose oxygen binding parameters are close to those of Hb Debrousse [10]. In Hb Debrousse, as well as in Hb Regina or Hb Saint Nazaire, it is likely that the structure of the heme pocket is only slightly disturbed. This is in contrast with what is observed in Hb Heathrow, where β103 phenylalanine is replaced by a leucine making a "hole" on this side of the heme [10]. Hb Debrousse also shares some properties with several unstable variants of this region, such as Hb Köln [β98 (FG5) Val → Met), in which the binding of the heme to the globin is weakened, leading to some degree of heme loss and instability in addition to the increased oxygen affinity [11].

In a patient suffering from a chronic well-compensated hemolytic anemia, such a variant will be found only if a careful hemoglobin investigation is performed. Unfortunately, such a study is usually done only when some complication occurs. In the case of Hb Debrousse, a severe anemia led to the discovery of the variant Hb: it was related to the association of a hemolytic process to a transient aplastic crisis induced by parvovirus B19 infection. These aplastic crises due to parvovirus B19 have been well documented in many situations in which a chronic hemolytic anemia exists, for example, sickle cell anemia, hereditary defects of the erythrocyte membrane proteins (chiefly hereditary spherocytosis), or RBC enzyme defects (see Harris [12] for review). To our knowledge, our observation is the first concerning an unstable Hb variant.

The diagnosis was more difficult in this case because this hemoglobin was a de novo case of a neutral variant not easy to evidence by electrophoretic methods. Hemoglobin variants resulting from de novo mutations are relatively frequent. Eighty-nine cases have been registered from data in the literature by Nute and Stamatoyannopoulos in 1988 [13]. This figure is certainly an underestimation, as these events are generally detected only when the mutated hemoglobin leads to a hematological disorder such as Heinz body hemolytic anemia, methemoglobinemia, or polycythemia. In addition, all cases of de novo mutation are far from being reported and, conversely, the identity of the parents is not always ascertained. These abnormal hemoglobins may result either from a somatic mutation in a very early stage of the embryological development of the propositus or from a parental origin with occurrence of a germline mosaicism [14,15]. In the case of Hb Debrousse, analysis of the DNA extracted from the spermatozoa of the father failed to demonstrate the presence of the mutation and make a possible recurrence risk improbable, unless the mother is a germinal mosaic.

ACKNOWLEDGMENTS

We acknowledge the skillful technical assistance of J. Riou (Laboratoire des Hémoglobines, Hôpital Henri Mondor, Créteil) and we thank Dr. L. Gebuhrer (Centre de Transfusion Sanguine de Lyon), who performed the HLA typing.

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